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IS THERE A CALCIFICATION FACTOR COMMON TO ALL CALCIFYING MATRICES?

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Abstract

This paper reviews the principal morphological findings pertinent to the early phases of the calcification process and explores the possibility that there may be a calcification factor common to all calcifying matrices. Three structures have a main role in calcification: collagen fibrils, matrix vesicles, and crystal ghosts. Only crystal ghosts are present in all calcified tissues, so that only they can be taken into consideration as a common calcification factor. They are organic molecules which have the same morphology as that of the inorganic structures present in the calcified matrix, which means that they can be considered as templates for those structures. Early calcification might be initiated by the binding of calcium and phosphate ions to the unmasked reactive groups of the crystal ghosts which are probably contained not only in the matrix, but also in the "holes" of the collagen fibrils and in matrix vesicles. The available data suggest that crystal ghosts share many of the properties of "crystal bound proteins". The involvement of alkaline phosphatase in their composition may account for their calcium- and phosphate-binding activity.

KEY WORDS: Calcification, Bone, Cartilage, Collagen fibrils, Matrix vesicles, Crystal ghosts, Crystals, Crystal associated organic material, Alkaline phosphatase

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Introduction

The calcification of biological matrices is induced and regulated by systemic and local factors. Of the latter, the structure, composition and cellular activity of the calcifiable matrix all play a leading role in the local deposition of calcium ions. The chemical composition and structural organization of matrices, and the types of cells they contain, vary between different tissues, so it might be supposed that each calcifiable matrix has its own calcification mechanism; but if analysis of this mechanism is strictly limited to its earliest phase and to the small areas where it is initially localized, it may reasonably be supposed that the basic processes and structures which induce and regulate calcium salt deposition are the same in all calcifiable matrices. To verify this hypothesis, an analysis should be performed on all the data available on the morphology and biochemistry of all calcified tissues. A survey of this type would inevitably be extremely complex. The scope of this paper has therefore been confined to the principal findings obtained by morphological investigations of the calcifiable mammalian tissues. When this procedure is adopted, it becomes apparent that the structures that are most probably involved in the earliest phase of the calcification process are collagen fibrils, matrix vesicles and crystal ghosts (Bonucci, 1984 a, 1985); proteoglycans, which are certainly involved in the process, seem to have an inhibitory role (De Bernard et al., 1977) and have not been considered in this review.

Background

For many years now, collagen fibrils have been considered the structures which induce and regulate the calcification process in bone (Glimcher, 1976). This concept emerged chiefly from the finding that collagen fibrils are the main constituents of the bone matrix, and from the ultrastructural evidence that in the early stages of the calcification process of compact, osteonic bone, or when it is incompletely calcified, there is a close relationship between collagen fibrils and the inorganic substance, which appears as electron-dense bands superimposed on the 40 nm bands of the collagen periodic pattern (Fig. 1) or, more exactly, on their hole regions. Later, as the calcification process moves towards completion, this close relationship is obscured by the development of needle- and filament-like structures (crystallites), whose amount is roughly proportional to the degree of calcification (Ascenzi et al., 1965).

On the basis of these and other observations it has been suggested that the calcification of the bone matrix and other biological matrices is due to a process of heterogeneous nucleation which occurs within the holes of the collagen fibrils, so accounting for the close relationship between the mineral substance and the periodic banding of the fibrils (Glimcher, 1959, 1976; Glimcher and Krane, 1968). On this theory, the calcification process occurs in delimited spaces (the fibril holes) where regularly located, reactive chemical groups appear to be responsible for the initial formation of inorganic nuclei which later grow to form the elongated crystallites typically found in bone matrix.

This theory is very satisfactory so far as compact lamellar bone is concerned, but less so for other types of bone. The close relationship between the mineral substance and the periodic banding of collagen is, in fact, only clearly recognizable in compact bone; it becomes less and less evident as the compactness of the collagen fibrils falls (in woven bone, for instance), and it is totally absent when the interfibrillary ground substance becomes prevalent over the collagen fibrils and when these are thin and loosely arranged (as in medullary bone of birds, for instance; Bonucci and Gherardi, 1975). Moreover, it is never recognizable in epiphyseal cartilage, where the collagen fibrils are thin and very loosely arranged. In these cases, the inorganic substance is arranged in needle- and filament-like crystallites from the beginning, and these are collected into roundish aggregates (calcification nodules). These crystallites, although oriented in some cases according to the axis of the collagen fibrils, are not related to them and lie in the interfibrillary ground substance (Fig. 2). The extrafibrillar localization of these crystallites is confirmed by the fact that in some cases they surround cross-sectioned uncalcified collagen fibrils, appearing as small rings encircling an empty space (Bonucci, 1984 a; Olson and Watabe, 1980; Schonborner et al., 1979).

These results show that, besides an intrafibrillar calcification which is prevalent in bone with the most compact collagen matrix, an extrafibrillar deposition of inorganic substance occurs to varying degrees in several types of bone with less aggregated matrix and represents the only way calcification can take place in tissues with a loose collagen texture such as medullary bone and epiphyseal cartilage. Thus the calcification process does not always occur within the holes of the collagen fibrils: if all calcified matrices are considered, it is more frequently found to be related to component(s) of the interfibrillar ground substance.

This finding conflicts with Glimcher's theory, according to which crystallites should derive from early inorganic nuclei formed in the holes and then grow between the collagen molecules inside the fibrils. This, however, should increase collagen solubility by interrupting intermolecular cross-links, whereas solubility decreases during calcification (Bonucci, 1971, 1984 a). To overcome this objection, Glimcher (1976) has suggested that crystallites grow not only within the holes, but also within "pores" present between collagen molecules; these pores, however, seem to be too thin to contain crystallites 2-4 nm thick without disrupting and destroying the inner order of fibrils. It is possible that the mineral substance contained within the holes of the fibrils remains in an "amorphous", finely granular condition (Bonucci, 1984

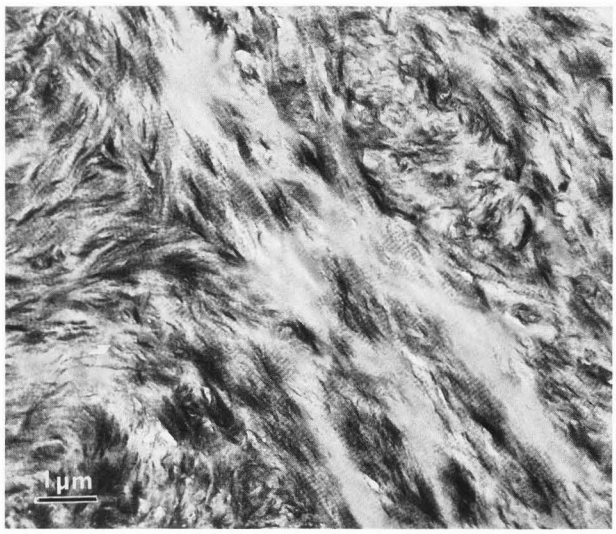

Fig. 1 - Incompletely calcified bone matrix: note that the mineral substance forms electron-dense bands which reinforce the collagen periodic banding. Unstained.

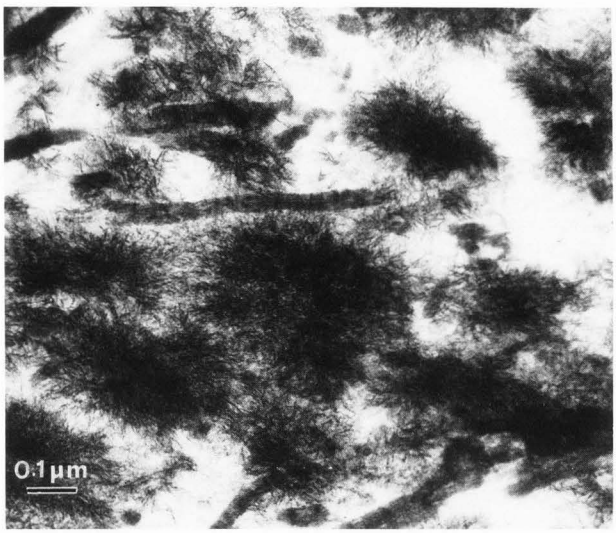

Fig. 2 - Detail of calcification front: calcification nodules and uncalcified collagen fibrils are visible. Unstained.

a) and does not grow into crystals which would inevitably disrupt the molecular organization of the fibrils. The crystallites seem to be formed and to grow only in the extrafibrillary space.

Without going any further into this problem, the available results show that calcification can occur outside the collagen fibrils, especially in tissues with a loose collagen texture. Consequently, the presence of the collagen fibrils in the matrix is not a necessary requisite for its calcification. These conclusions are in agreement with the observation that calcification can occur in matrices which do not normally contain collagen fibrils (enamel, for instance) and even in pathological bone matrix abnormally synthesized without collagen fibrils (as in fibrogenesis imperfecta ossium; Bonucci, 1984 a). It is evident that collagen fibrils as such cannot constitute a calcification factor common to all calcified tissues; if such a factor exists, it must be found in some specific substance(s) present both in the holes of the collagen fibrils and in the interfibrillary ground substance.

Morphological investigations

The search for one or more such substances was carried out by electron microscopy and conventional and ultrastructural histochemistry of calcifying cartilage (Bonucci, 1967, 1969, 1970, 1971, 1978, 1981), different types of bone (Bianco et al., 1985; Bonucci, 1971; Bonucci and Gherardi, 1975; Bonucci and Silvestrini, 1984), developing enamel (Bonucci, 1984 a; Hayashi et al., 1986), and pathologically calcified tissues (Bonucci, 1978), including calcified mitochondria (Bonucci et al., 1973). These investigations led to the discovery of matrix vesicles and crystal ghosts.

Matrix vesicles

The term matrix vesicle refers to roundish bodies (Fig. 3), between 25 to 200 nm in diameter, consisting of homogeneous and amorphous, PAS-positive and osmiophilic matrix surrounded by a membrane (Anderson, 1967, 1969; Bonucci, 1967, 1970). They are particularly frequent in the longitudinal septa of the epiphyseal cartilage, where they are mainly localized around the chondrocytes. They have no direct connection with the cells, though, as shown by serial sections (Bonucci, 1970, 1978) and by the fact that they can be isolated from other matrix components by differential centrifugation (Ali et al., 1971). Histochemical and biochemical investigations have shown that they contain glycoproteins and lipids, are surrounded by acid proteoglycans (Bonucci, 1970) and that - most important of all -

they have a strong alkaline phosphatase activity (Ali et al., 1970, 1971; Fortuna et al., 1978; Kahn et al., 1978; Matsuzawa and Anderson, 1971; Meikle, 1976; Väänänen and Korhonen, 1980).

The matrix vesicles are of cellular origin, as shown by the membrane around them. In epiphyseal cartilage they are formed by the fragmentation of cell processes, detachment of the swollen tip of cell processes, and fragmentation of whole chondrocytes (Bonucci, 1970, 1984 b). Their origin in bone is less certain and a process of exocytosis is possible (Bonucci and Silvestrini, 1984).

The main reason why matrix vesicles are of interest is the fact that they represent the locus of initial calcification. Electron microscopy, especially when carried out on serial sections (Bonucci and Dearden, 1976), clearly shows that in epiphyseal cartilage the earliest aggregates of crystallites are formed within matrix vesicles and on their membrane (Fig. 3). As calcification proceeds, these aggregates grow in size until the whole vesicle is calcified and then spread from the vesicle into the surrounding matrix, forming a calcification nodule. These observations, which have also been reported for calcifying cartilage prepared by ultracryomicrotomy and other nonaqueous techniques (Gay, 1977; Gay et al., 1978; Hunziker et al., 1981; Schraer and Gay, 1977), show that matrix vesicles have the property of initiating the calcification process - a conclusion confirmed by the observation that they concentrate calcium ions *in vitro* (Ali and Evans, 1973) - and that *in vivo* this concentration rises in moving from the hypertrophic to the calcifying zone of the epiphyseal cartilage (Brighton and Hunt, 1978).

Matrix vesicles can be found in bone (Bonucci, 1971), especially in the embryonic, trabecular, and medullary bone; however, very few or none of them are present in compact, osteonic bone. They are also present in so-called "mantle" dentin, but not in the "circumpulpal" dentin that is formed later (Bonucci, 1984 b). Moreover, they have been described in many types of pathologically calcifying soft tissues (Anderson, 1976).

Matrix vesicles might be considered good candidates for the role of a calcification factor common to all calcifying matrices because they are present in most of them. However, their frequency changes in different types of bone and, above all, they are not present in enamel. Consequently, what has been previously said about collagen fibrils can be repeated now: matrix vesicles are not present in all calcifying matrices, and cannot be considered a calcification factor common to those matrices. If there is such a factor, it could not consist of matrix vesicles themselves, but of one or more substances present in their matrix.

The spread of the calcification process from matrix vesicles into the surrounding matrix, which probably involves the dissolution of the vesicles and their membrane (Bonucci, 1981), gives rise to the formation of calcification nodules, i.e., roundish aggregates of crystallites (Figs. 2, 3). As calcification progresses, the nodules grow through the addition of new crystallites and eventually coalesce, so masking the whole matrix. At this stage, an electron microscope study of the calcified organic matrix is practically impossible, because the inorganic substance completely masks the organic components. On the other hand, the unmasking of these components by conventional decalcification induces several artifacts, mainly due to extraction of many organic substances. These artifacts can be avoided by using the special method of decalcification called post-embedding decalcification and staining (PEDS; Bonucci, 1967, 1969, 1984 a; Bonucci and Reurink, 1978), which consists in decalcifying the specimens after embedding them in an epoxy resin.

Crystal ghosts

The PEDS method shows that in all calcified matrices the removal of the inorganic substance makes it possible to stain organic, needle- and filament-like structures (Fig. 4) which, because they have the same morphology as untreated crystallites, have been called crystal ghosts (Bonucci, 1967, 1969, 1971, 1975).

Crystal ghosts, or structures resembling them, have been found in all the calcified matrices that have been decalcified and stained using the PEDS method. They have been described in cartilage (Appleton, 1971; Bonucci, 1967, 1969, 1971; Smith, 1970), bone (Bonucci, 1971; De Bernard et al., 1980), enamel (Bai and Warshawsky, 1985; Bishop and Warshawsky, 1982; Bonucci, 1984 a; Frank, 1973; Hayashi et al., 1986; Nanci et al., 1983; Rönnholm, 1962; Smales, 1975), peritubular dentine (Goldberg et al., 1978), apical

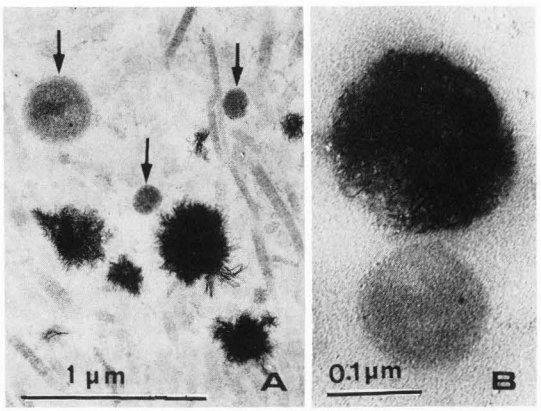

Fig. 3 - A: Uncalcified matrix vesicles (arrows) and calcification nodules probably corresponding to calcified matrix vesicles. Uranyl acetate and lead citrate. B: Detail of two matrix vesicles, one of which is completely calcified. Unstained.

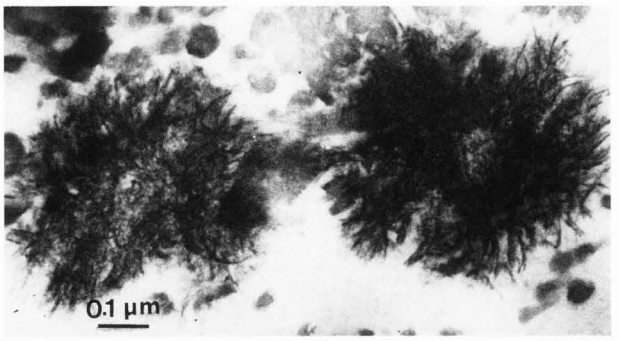

Fig. 4 - Aggregates of crystal ghosts corresponding to two decalcified and stained calcification nodules. PEDS (uranyl acetate and lead citrate after formic acid decalcification).

cementum (Hayashi, 1985), and extraskeletal calcified tissues (Bonucci et al., 1973; Davis et al., 1981).

Crystal ghosts are most easily recognizable in areas of early calcification (small calcification nodules) and at the borders of calcified areas. They are digested by papain (Bonucci, 1969) and react with chromium sulphate (Appleton, 1971), phosphotungstic acid (Bonucci, 1969), and bismuth nitrate (Smith, 1970). Moreover, the areas which contain crystal ghosts can be histochemically stained with Alcian blue at pH 1.8 and with Sudan black B according to Irving (Bonucci et al., 1978). Moreover, these areas contain acidic proteoglycans, as is shown by the presence of sulfate groups (Davis et al., 1982; Groot, 1982).

All these results show that the crystal ghosts are composite organic structures which contain vic-glycol and acidic groups and probably have a lipidic component. Because they exactly reproduce the morphology and arrangement of untreated crystallites, it could be hypothesized that they act as templates for crystal formation. The recent observation that in developing enamel the crystals develop in continuity with crystal-like fibrillar components of the stippled material, so that these latter seemed to be transformed into the former (Sakakura, 1986), is in agreement with this hypothesis. Because crystal ghosts are observed in all calcifying matrices, they might represent the calcification factor common to all calcifying tissues discussed in this paper. This, however, does not mean that they always have the same chemical composition; it cannot be excluded, in fact, that collagen fibrils, interfibrillar matrix, and matrix vesicles contain chemically different crystal ghosts having common Ca-binding groups which, after all, would represent the actual common calcification factor.

It is interesting to note that the morphologically demonstrable crystal ghosts share many features with the "crystal bound proteins" which have been isolated by using biochemical methods in bone and enamel. In particular, this is true of osteocalcin (Hauschka et al., 1975) and osteonectin (Termine et al., 1981) from bone, amelogenins and enamelins from enamel (Termine et al., 1980; Lyaruu et al., 1982), phosphoprotein from dentin (Zanetti et al., 1981), and chondrocalcin from cartilage (Poole et al., 1984). Just as crystal ghosts are linked to crystallites, all these proteins are closely linked to the inorganic substance. It may be speculated that they are components of the crystal ghosts, a hypothesis supported by recent immunohistochemical investigations on osteonectin and enamelin distribution in bone and enamel (Bianco et al., 1985; Hayashi et al., 1986).

Of these calcium-binding proteins,

one is of particular interest - a glycoprotein recently isolated from matrix vesicles (De Bernard et al., 1985) which displays alkaline phosphatase activity and calcium binding properties. In fact, because it can hydrolyze phosphate esters and simultaneously link calcium ions, there are good grounds for considering it responsible for the induction of the earliest phase of the calcification process. Moreover, considering that it interacts in vitro with proteoglycan subunits and type II collagen in epiphyseal cartilage (Vittur et al., 1984), it might also orient the deposition of calcium phosphate in conformity with the structures of the matrix. Immunohistochemical studies on the localization of this phosphatase in the growth zones of the epiphyseal cartilage show that it is present in the membrane of the maturing and hypertrophic chondrocytes, in the matrix vesicles, in the uncalcified matrix of the intercellular longitudinal columns, and in the calcified matrix, and that it has a strong enzymatic activity in all these sites except the calcified matrix, where it seems to be buried in the inorganic substance and inactivated (De Bernard et al., 1986). On the basis of these observations it could be speculated that this protein induces calcium phosphate precipitation because of its enzymatic activity and calcium-binding properties, which account for its incorporation in, and consequent masking by, the inorganic substance. This is exactly the behaviour one would expect if this alkaline phosphatase was a component of the crystal ghosts.

Conclusions

The morphological observations have the intrinsic limitation of being static, so that a kinetic or temporal outline of the calcification process based on them is necessarily subjective. However, considering the different results obtained at different stages of the process, the following conclusions can be suggested.

Morphologic investigations have shown that three main structures are involved in calcification, i.e., collagen fibrils, matrix vesicles, and crystal ghosts. Only crystal ghosts, however, are present in all calcifying matrices. Although the presence of a structure does not mean that it is involved in calcification, and although the crystal ghosts have not been isolated chemically so that their calcifying properties have not been tested directly, their constant presence in calcification nodules and their direct connection with crystallites cannot be casual. In this connection, it cannot be disregarded that they share many characteristics of the crystal bound proteins, whose calcium-binding properties are indubitable. On the basis of the available data it can be suggested that the crystal ghosts are components of the matrix and that, before the calcification process begins, they are inhibited by some substance (possibly proteoglycans) whose partial or complete removal unmasks their reacting groups, which can bind calcium ions. It follows that early calcification should not be considered as a purely physical process of precipitation occurring in preformed spaces (holes of collagen fibrils and matrix vesicles), but as a chemical process induced and regulated by organic molecules (primarily, alkaline phosphatase) which are contained in the matrix, in the holes of collagen fibrils and in matrix vesicles and which, once unmasked and made reactive, acquire calcium-binding properties. In doing so, they remain more or less completely embedded in the crystallites, whose shape repeats that of the template molecule. Although this line of reasoning is partly speculative, it is in accordance both with the available morphological findings, and with the biochemical observation that not only the crystal bound proteins, but lipids also (Odutuga and Prout, 1974; Shapiro, 1970; Wuthier, 1968), are so closely linked to the inorganic substance that they can only be extracted from the calcified matrices after decalcification.

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Discussion with Reviewers

S.R. Khan: Please define crystal ghosts for me. How are they formed? Where do they come from? Would crystal ghosts of one system differ from crystal ghosts of the other systems?
Author: Crystal ghosts can be defined as organic (probably glyco-lipo-proteic) molecules whose chemical groups, once unmasked and made reactive, link calcium and/or phosphate ions thus functioning as templates of crystal-like, organic-inorganic structures which repeat their shape. Probably, they are components of the calcifying matrix, as shown by the experiments of Smith (1970) with bismuth nitrate, and are made reactive by removal of inhibitor(s). It is not known where they come from; however, acid proteoglycans seem to be involved in the formation of crystal ghosts of epiphyseal cartilage, as shown not only by the histochemical properties of the ghosts, but also by the recent observation (unpublished) that in areas of early calcification part of the crystal ghosts are connected with, and protrude from the dense granules which, under the electron microscope, represent the acid proteoglycans of the cartilage matrix. In enamel, they might be components of the so-called stippled material as suggested by the observation of Sakakura (1986) in that the enamel crystals develop in continuity with fibrillar components of the stippled material so that the latter seem to be transformed into the former. This seems to show that the crystal ghosts of one system differ from those of other calcifying systems.

A.S. Posner: Why must there be a common factor in all calcifying systems? Isn't it possible for different factors to be active in enamel and bone, for example?
Author: This question concerns the philosophy of the present investigation. Obviously, it is possible that different factors are active in different systems. However, structures which are components of all calcified tissues, as crystal ghosts are, are good candidates for having the same effect on the calcification process of all of them. It must be underlined, however, that the term crystal ghost refers to morphologically identifiable structures which may not necessarily have an identical chemical composition in different calcifying tissues.

R.L. Hackett: If the crystal ghosts are a unifying factor, they should also be present in other mineralizing systems such as calcium carbonate in marine animals or calcium oxalate in human kidney stones. Please comment.
Author: It is probably hazardous to com-

pare the calcification process of vertebrates with that of invertebrates or pathologically formed stones. However, it is interesting that intracrystalline organic material grossly comparable to crystal ghosts, but not necessarily similar from a chemical point of view, has been found both in invertebrate skeletons (see The Mechanisms of Mineralization in the Invertebrates and Plants, N. Watabe, K.M. Wilbur (eds). Univ. S. Carolina Press, Columbia 1976) and in oxalate stones (Khan S.R., Hackett R.L. (1985) Developmental morphology of calcium oxalate foreign body stones in rats. Calcif. Tiss. Int. 37: 165-173).

E.P. Katz: I am not aware of any findings demonstrating that crystal ghosts are preformed. How can one distinguish this case from ghosts postformed on preexisting crystals?

H.C. Anderson: How can you be certain that "ghosts" are not formed by adherence of organic material to pre-formed apatite crystals without the ghost material serving as the initial template or instigator of crystal formation? Has it ever been shown that the local deposition of crystal ghosts precedes the appearance of superimposed material (as has been shown in the case of matrix vesicles)?

S.Y. Ali: Is it not far more likely that some of the proteins and organic compounds that bind to the fine crystallites are therefore a morphological consequence of calcification rather than an initiating calcification factor?

A.S. Posner: Isn't it possible that the crystal ghost is deposited after crystal deposition and growth?

S.R. Khan: Since crystal ghosts have so far been identified in calcified matrices only, it is possible that crystal ghosts represent organic substances present in the matrix that adsorbed on the crystal surface and thus crystal ghosts may be a result rather than a cause of crystal initiation. This would explain their shape conforming to the shape of the crystals and the presence of a range of organic molecules from lipids to various types of "crystal bound proteins".

Author: These questions are similar and can be answered together. They concern two principal problems: 1) are the crystal ghosts preformed in the matrix; 2) if they are not preformed, might they be formed by adherence of organic material to preexisting crystallites either as a natural phenomenon occurring during the calcification process or as an adsorption artifact due to fixation, dehydration and/or embedding?

As far as the first problem is concerned, it must be remembered that the crystal ghosts of cartilage, bone and enamel appear as filaments only because, once calcified, they become rigid structures which can resist the distorting action of fixation, dehydration and embedding. In fact, electron microscopy of specimens decalcified before embedding shows that only a rather amorphous organic material is present in place of the decalcified calcification nodules. Consequently, in case the crystal ghosts were present before calcification, they could be greatly modified by fixation, dehydration and embedding and could be hardly recognizable. Such a type of distortion occurs in proteoglycan molecules which, under the electron microscope, appear as $BiNO_3$ stained "dots" although their actual shape is that of ramified filaments (see Smith, 1970).

As far as the second problem is concerned, that is, that the crystal ghosts might be formed by adherence of organic material to preexisting crystallites, it must be considered that if the adherence phenomenon is due to the presence of hydroxyapatite, it should occur soon after the earliest mineral aggregates are formed. This might have two opposite effects: either it might inhibit any further crystal growth by completely surrounding and coating the growing crystal surfaces, a possibility which is excluded by the fact that the crystallites have a length of some ten nm; or it might be a step of an active process in which the adherence of Ca-binding proteins to the earliest mineral aggregates induces further Ca-accretion to them and favours crystal growth. Further investigation is needed to ascertain if this possibility can really occur.

The other possibility (that adherence of proteins to preformed crystallites occurs by an adsorption artifact during specimen preparation) is improbable because fixation and dehydration denature the organic molecules and denaturation eliminates protein adsorption on hydroxyapatite crystals. Moreover, it must be

stressed that the crystal ghosts are not removed by fixing solutions containing 1.2M phosphate buffer, which completely desorb organic material from hydroxyapatite. Finally, it should be considered that, once decalcification had removed the crystallites, their coat of adsorbed organic material, if present, would appear as hollow cylinders, tubules, or paired profiles when sectioned according to their long axis, and as rings when sectioned transversally (as it happens for mature enamel crystallites), whereas the crystal ghosts always have a full structure and at high enlargement show a solid helical conformation of the kind to be expected of protein molecules.

A.S. Posner: The author says that the crystal ghosts are the size and shape of mature crystals and are involved in the deposition of the crystals. How does this view fit in with the observation that early crystals are smaller in size than more mature crystals?
Author: Actually, crystal ghosts are the size and shape of immature crystals, that is, crystals at the beginning of their formation. This is chiefly true for immature enamel, whose crystal ghosts have the same filamentous or ribbon-like shape as that of the early crystallites. The morphological evidence of crystal ghosts decreases as the crystals mature; for instance, they are easily recognizable at the periphery of a calcification nodule, but not or seldom in its center. This can be due to the fact that during calcification they may be progressively obscured or lost to view as a consequence of reduction of organic material, or they may be cross-linked, with consequent reduction of chemical groups responsive to the stains employed in electron microscopy.

S.Y.Ali: Your concept implies that matrix vesicles are not a universal calcifying factor only because they are not found in enamel, but do you believe that enamel is a specialised tissue unlike the mesenchymal tissues that you have listed? If the cells in enamel are different and the matrix protein (keratin) is different will not the mechanism of calcification be different?
H.C. Anderson: Matrix vesicles have been isolated and shown capable of initiating calcification with alacrity. The fact that matrix vesicles are not seen in densely mineralized bone is hardly surprising inasmuch as they tend to be disrupted and destroyed in the early stages of calcification. Furthermore, matrix vesicle action need not be invoked to explain enamel mineralization, because vesicle-activated mineralization of the pre-dentin precedes mineral-deposition in enamel and is contiguous with the enamel so that mineral proliferation could be propagated from dentin to enamel without requiring reinitiation by matrix vesicles.
Author: Enamel is certainly different from cartilage and bone. It is considered here chiefly because the aim of this paper was to look for a factor common to all calcifying tissues and enamel could not be disregarded. On the other hand, the presence of filament- and ribbon-like crystal ghosts during the early phase of enamel formation clearly shows that under this particular point of view it is similar to other calcifying tissues. I know that it has been suggested (but not proved) that enamel mineralization might be activated by the already calcified dentin, so that matrix vesicles might not be necessary for enamel calcification. However, the statement that matrix vesicles cannot be a common calcification factor is supported by the observation that calcification of compact osteonic bone also occurs without the intervention of matrix vesicles which are lacking in this kind of tissue. This lack of vesicles can hardly be explained by their rapid destruction and disappearance during calcification, because many of them are present in other types of bone (embryonal bone, for instance) which calcify more rapidly than osteonic bone.

S.Y. Ali: Would it not be true to say that because you are applying the same decalcification technique (post-embedding decalcification and staining) to all the calcified tissues, that you have mentioned, that the common factor is the artefact of PEDS technique rather than a universal calcification factor?
Author: The PEDS technique is very simple and is carried out when the tissue, being embedded in an epoxy resin, is rigid and cannot be distorted. I recall that crystal ghosts, or intracrystallite organic structures similar to them, have been found by Smales (1975) in enamel crystallites using a different decalcification technique and

this would seem to lend credence to this technique and provide additional evidence that these structures are not artifacts.

S.Y. Ali: I suspect that the PEDS technique with formic acid does not decalcify the nodules completely and therefore you are restaining the same outlines when you introduce uranyl acetate and lead citrate. Have you any evidence to indicate that the section is completely decalcified? Have you done electron probe analysis of the decalcified sections?
Author: It is well known that ultrathin sections are decalcified even if left floating on distilled water for a few minutes (Boothroyd B. (1964) The problem of demineralization in thin sections of fully calcified bone. J.Cell Biol. 20, 165-173). Sections floated on formic acid or EDTA are fully decalcified, as shown by the von Kossa method under the optical microscope and, under the electron microscope, by the complete disappearance of the crystallites in decalcified-unstained sections (with reappraisal of crystal ghosts in decalcified-stained sections) and by the change to an amorphous pattern in electron diffraction. Electron probe analysis of the decalcified sections has not been done.

H.C. Anderson: Has it ever been shown that the substituents of crystal ghosts do have biological activity which can promote mineralization, or regulate mineralization in any way?
Author: No, it has not, because the constituents of the crystal ghosts are not exactly known.

H.C. Anderson: Can it be shown in a convincing way that Ca-binding alkaline phosphatase is a substituent of the crystal ghosts?
Author: In collobaration with De Bernard and his group (De Bernard et al., 1986) it has been shown by ultrastructural immunohistochemistry that anti-alkaline phosphatase antibodies react with calcification nodules. Further personal investigations (unpublished) have shown that the same reaction occurs with the organic material unmasked by decalcification in the calcification nodules. Unfortunately, the method used for alkaline phosphatase demonstration does not allow satisfactory demonstration of crystal ghosts.

S.Y. Ali: I accept that the alkaline phosphatase and the lipid are matrix vesicle derived and therefore they would be present in the core of the calcification nodule (crystal ghosts). We have occasionally observed matrix vesicles in the centre of a calcification nodule when it is partly decalcified in hot uranyl acetate and when the plane of the section goes through the central core of the calcifying nodule. Have you seen matrix vesicles in such centrally traversed calcifying nodules (crystal ghosts)?
Author: Roundish areas of electron density are sometimes present in the central part of the aggregates of crystal ghosts; they might be remnants of disrupted matrix vesicles. Membranes, or fragments of membranes, have never been found.

S.Y. Ali: Do you appreciate that by saying that the crystal ghosts may be composed of "acidic glyco-lipo-proteic components" that you have covered most of the major organic molecules in biochemistry apart from nucleic acids? How can we use the scientific method to disprove the existence of a chemical complex with such a wide ranging composition?
Author: If I knew the answers, probably most of the problems about crystal ghosts would have been solved already. I can only say that the experimental evidence shows that the crystal ghosts can be digested by papain, as proteins can, react histochemically with acidic phosphotungstic acid (PTA), as glycoproteins do, and with cations, as acid proteoglycans do, and are contained in areas which are positive with Irving's method for masked lipids.

S.R. Khan: You have not discussed the role of calcium-phospholipid-phosphate complexes and proteolipids. Could crystal ghosts be the morphological equivalent of these biochemical entities?
Author: The possibility that phospholipids can form complexes with calcium and phosphate ions and initiate the calcification process is in line with the observation that lipids are closely bound to the inorganic substance, and that crystal ghosts are present in areas which can be stained with Irving's method for masked lipids. However, it will not be possible to provide an answer to your question until crystal ghosts are isolated and examined

by biochemical methods.

S.R. Khan: Would crystal ghosts also be involved in pathological calcification?
Author: The answer is yes if the concept of crystal ghost is extended to include every substance which has calcium binding properties and can work as crystal template. For instance, the intramitochondrial clusters of mineral substance found in mitochondria of hepatocytes of rats intoxicated with carbon tetrachloride are intimately linked to an organic substratum whose morphological and histochemical properties are similar to those of the crystal ghosts of cartilage, bone and enamel.

H.C. Anderson: Are there any calcification diseases in which deficient, superfluous or abnormal ghost material was shown to be associated with abnormal or defective mineralization? Such an "experiment of nature" might help to substantiate a role for crystal ghosts in mineralization.
Author: No, to my knowledge, there are no such diseases. However, I would like to recall again the interesting paper of Sakakura (1986) because it provides pertinent information on this question. Sakakura has found that a great quantity of stippled material accumulates between the enamel and ameloblasts in *in vitro* culture of mouse embryo molars. Although it cannot be stated that this material is equivalent to crystal ghosts, it does contain an accumulation of filaments "similar to enamel crystals", and consequently similar in appearance to crystal ghosts.